Research Article

Received: 20 February 2008 Revised: 16 September 2008 Accepted: 1 October 2008 Published online in Wiley Interscience: 19 November 2008

(www.interscience.com) DOI 10.1002/jsfa.3453

Physical distribution and characteristics of meat and bone meal protein†

Rafael A Garcia[a]* and John G Phillips[b]

Abstract

BACKGROUND: Use of meat and bone meal (MBM) in animal feed has been progressively restricted. Production of an MBM protein isolate would facilitate the development of new applications. Rational design of a process to produce such an isolate requires knowledge of MBM protein distribution and characteristics.

RESULTS: MBM consists of varying proportions of bone and soft tissue particles, but the soft tissue particles contain >80% of the protein. Under mild conditions only a small portion of MBM protein can be solubilized; the most aggressive non-hydrolytic conditions fail to solubilize >55% of the protein. Amino acid analysis reveals that collagen accounts for approximately 17% of the protein in soft tissue particles and 26% of that in bone particles. The extractable fractions of MBM protein are highly polydisperse and have weight-average molecular masses of 71.1–86.7 kDa.

CONCLUSION: MBM protein is a challenging substance to utilize functionally, largely due to its low solubility and intermingling with non-protein substances. Results suggest that production of an MBM protein isolate will have to incorporate limited hydrolysis.
Published 2008 by John Wiley & Sons, Ltd.

Keywords: meat and bone meal; protein isolate; characterization; non-food application; collagen

INTRODUCTION

Meat and bone meal (MBM) is a high-protein commodity produced by the rendering of fat from unmarketable animal tissue, primarily the bones and offal from slaughtered livestock, the carcasses of deadstock, and retail meat products that have exceeded their 'sell-by' dates.[1] Concerns related to bovine spongiform encephalopathy have progressively restricted MBM's conventional use as a feed ingredient. Consequently, significant attention has focused on the development of new applications for MBM.[2–4] Other than use in industrial fuel applications, however, no new applications have been commercially adopted.

Most of the proposed applications for MBM would utilize the protein that comprises about 50% of MBM. Proteins isolated from other agricultural commodities such as soy, milk, and cattle hide have been utilized successfully in non-edible applications. Soy protein adhesives are beginning to replace synthetic adhesives in manufactured wood products;[5,6] protein recovered from leather-making waste (known as *chrome shavings*) is used commercially as a technical-grade gelatin;[7,8] biodegradable films made from milk proteins are poised to become important in commercial packaging.[9,10] These applications, however, developed primarily after each protein was available in a relatively concentrated, homogeneous and soluble form. Soy adhesives, for example, were developed from soy protein concentrate or soy flour, rather than directly from the soybeans.

Rational design of a process for producing such a protein isolate requires knowledge of the host biological matrix, the physical distribution of the protein in the matrix, and the properties of the protein itself. The present study examines these aspects of MBM with the intent to facilitate future process design.

MATERIALS AND METHODS

MBM samples were obtained from 18 rendering facilities in the USA and Canada through the Fats and Proteins Research Foundation (Alexandria, VA, USA) and provided to the researchers without revealing the firms' identities, as described previously.[1] For the present study, commercially recognized subtypes of MBM, including poultry meal and meat meal, were not differentiated from MBM. Samples were specifically requested to be from a single source, i.e., not a blend of MBM from multiple processing lines or different plants. The anonymous manufacturers provided detailed information on their raw material and processing method.

Subsampling and sample preparation

Individual MBM particles vary widely in size and composition, and the particle types have a strong tendency to spontaneously segregate, so care was required to obtain small, representative samples for analysis. An 80 g subsample was taken from each bulk sample by repeated cone and quartering. This subsample

* *Correspondence to: Rafael A Garcia, USDA, ARS, Eastern Regional Research Center, Fats, Oils and Animal Coproducts Research Unit, Wyndmoor, PA 19038, USA. E-mail: Rafael.Garcia@ars.usda.gov*

† *Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.*

a *USDA, ARS, Eastern Regional Research Center, Fats, Oils and Animal Coproducts Research Unit, Wyndmoor, PA 19038, USA*

b *USDA, ARS, North Atlantic Area Office, Wyndmoor, PA 19038, USA*

was defatted in standard Soxhlet extractors for 4 h using hexane. The bone particles of this defatted material were separated from the soft tissue particles using a heavy-fluid method adapted from Mendez and Dale.[11] Approximately 4 g MBM and 80 mL chloroform were added to a graduated cylinder and stirred to break apart any multi-particle clumps. This suspension was allowed to settle for 5 min, during which a large majority of the soft-tissue particles floated to the surface and the bone particles sank to the bottom. The top layer of particles and the liquid were then filtered through Whatman #50 filter paper in a Büchner funnel, leaving behind undecanted bone particles. From this point forward, the soft-tissue and bone fractions were treated in parallel. The particles were milled in a freezer mill (model 6800, Spex Centiprep Inc., Metuchen, NJ, USA), to the point where at least 95% of the sample would pass through a wire mesh sieve with 710 µm openings.

Proximate analysis

Nitrogen content was determined in six replicates using an automated analyzer (model FP-2000, Leco Corporation, St Joseph, MI, USA) according to the manufacturer's directions; crude protein is reported as percent nitrogen × 6.25. Ash determination was performed in triplicate by overnight incineration of 2 g samples in ceramic crucibles in a 600 °C muffle furnace.

Hydroxyproline analysis

Bone samples (7 mg) were demineralized prior to analysis, by extracting for 40 h at 10 °C with 0.5 mol L^{-1} EDTA and a bacteriostat. The extract was passed though a PD-10 desalting column (GE Healthcare, Piscataway, NJ, USA) to remove the EDTA and complexed mineral. The desalted extract and remaining solid portion of the bone sample were combined and dehydrated using a rotary evaporator.

Soft-tissue samples (5 mg) or the demineralized bone samples were hydrolyzed using a PicoTag workstation (Waters Corporation, Milford, MA, USA) following the directions in the owner's manual. Briefly, 10 mL constant-boiling HCl and a single crystal of phenol were added to a glass hydrolysis flask containing the analytical sample. The flasks were repeatedly evacuated and flushed with nitrogen and then incubated for 20 h at 110 °C. Small portions of the resulting hydrolysate were passed though a filter with 0.2 µm pores and dried under vacuum.

Hydrolyzed samples were derivatized with AccQFluor reagent (Waters) according to the manufacturer's directions. Chromatography was performed using procedures described as 'mixture 1' in van Wandelen and Cohen[12] and involved the use of four separate eluents. Separation was achieved using an AccQTag C_{18} reverse-phase column (Waters); detection by fluorescence used excitation with 250 nm light and measured emission at 395 nm.

Extraction of soluble protein

Small sample portions (6 mg soft tissue or 12 mg bone) were extracted at 6 °C overnight, with either a mild or an aggressive protein-solubilizing solution, described in Table 1. The resulting extracts were treated for 4 min with a microtip ultrasonic probe (Misonix model 3000, Farmingdale, NY, USA) set at intensity 5, 50% duty cycle, while sitting in an ice bath. Protein concentration was determined in triplicate with an assay specifically designed to be compatible with reductants, detergents and chelators (RC DC Protein Assay, Bio-Rad, Hercules, CA, USA) using bovine γ-globulin as the standard. Bio-Rad's ReadyPrep 2-D Cleanup Kit was used to concentrate samples, remove contaminants and exchange the extraction solution for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Table 1). In this process, the protein concentrations of individual samples were adjusted to be roughly equal to one another by varying the volume of loading buffer used to redissolve the pellet. After redissolving, samples were incubated at 95 °C for 10 min. The loading buffer contained either β-galactosidase or α-lactalbumin, depending on the type of SDS-PAGE gel to be used, as an internal standard.

SDS-PAGE

Extracts were analyzed by SDS-PAGE using the Phastsystem (Amersham Biosciences, Piscataway, NJ, USA). Phastgels with either an 8–25% concentration gradient or a homogeneous 20% concentration along with 30% ethylene glycol (referred to as 'high-density' gels) were used according to the manufacturer's protocol, except that an initial low-current step was added to the electrophoresis program, to reduce streaking. A blank lane was included on each gel to provide data on background signal. The gels were stained overnight with SYPRO Ruby Protein Gel stain (Sigma, St Louis, MO, USA) according to the manufacturer's directions, except that the gels were fixed with a 12.5% gluteraldehyde solution. It has been reported that SYPRO

Table 1. Extraction and analysis solution compositions

	Mild extraction solution	Aggressive extraction solution	SDS-PAGE loading buffer
pH	8.0	8.0	8.0
Tris (mol L^{-1})	0.01	0.01	0.01
EDTA (mol L^{-1})	0.001	0.001	0.001
Protease inhibitor cocktail (% v/v)	0.1	0.1	0.1
Sodium azide (% w/v)	0.05	0.05	0.05
Sodium chloride (mol L^{-1})	0.1	–	–
Urea (mol L^{-1})	–	7	7
Thiourea (mol L^{-1})	–	2	2
Dithiothreitol (mol L^{-1})	–	1	1
Sodium dodecyl sulfate (% w/v)	–	2.5	2.5
β-Galactosidase from *E. coli* or bovine α-lactalbumin (% w/v)	–	–	5
Bromophenol blue	–	–	Adequate to color

Ruby staining of SDS-PAGE gels gives a linear response to protein concentration for over three orders of magnitude[13] and that overnight staining, as used in the present study, optimizes the linearity of response.[14] Fluorescence densitometery was performed on an FLA-5000 fluorescent image analyzer (Fujifilm, Tokyo, Japan) at 16 bits, 25 µm resolution, using a 473 nm laser.

All chemicals used were of reagent or molecular biology grade.

Gel data analysis

Gel images were analyzed using functions in both Multi Gauge v2.02 (Fujifilm, Tokyo, Japan) quantitation software and Excel v11 (Microsoft Corporation, Redmond, WA, USA). Briefly, each gel lane was analyzed with a tool that determined average fluorescence intensity *versus* distance from a defined origin. Data from a blank lane were subtracted from each sample lane to correct for background fluorescence. Integrated area of the internal standard peak was used to correct for inaccuracies in sample volume applied to the gel.

RESULTS AND DISCUSSION

Physical distribution of MBM protein

Individual MBM particles may originate in almost any organ in an animal's body, and consequently any two given MBM particles may differ substantially in their composition or other properties. Qualitatively, two general classes of particles are easily recognizable: hard, white particles presumably made of bone, and softer, brown particles presumably made of soft tissue. A small minority of particles do not fit well into either class; these include bits of plastic or small stones as well as tissue particles that appear to be intermediate between the two classes.

The mass proportions of soft tissue to bone vary widely in the samples tested (Table 2); the soft tissue fraction ranged from 36.49% to 73.90%. Among samples that were made from ≥75% by mass of a single species, samples made from cattle tissue had a significantly ($\alpha = 0.05$) greater proportion of bone (49.62%) compared to swine (33.96%); the sole chicken sample had a relatively low proportion of bone (39.20%). A known allometric relationship between body mass and skeletal mass[15] probably accounts for some of this difference; the ratio of skeletal mass to total body mass is greater for large animals, such as cattle, than it is for smaller animals, such as swine or poultry. Differences in butchering practices (i.e., which tissues are harvested) may also play a role. Samples in which no single species made up ≥75% by mass had significantly ($\alpha = 0.05$) less bone content (31.94%) compared to single-species samples (43.24%). Rendering plants that process almost entirely one species tend to be closely tied to a nearby meat-packing plant; meat-packing plants are very efficient at harvesting whatever soft tissue they can sell profitably. Rendering plants processing a wider variety of species may be processing more whole animal deadstock and other material such as unsellable retail meat, both of which would depress the bone-to-soft tissue ratio. While the composition of the unblended samples tested here varied considerably, it should be noted that buyers of MBM specify acceptable ranges of various parameters, including protein and ash content, and it is standard practice for renderers and feed mills to meet these specifications by blending MBM from different sources.

Table 2. Relative mass of bone and soft tissue fractions in dry, defatted, unblended MBM, by species. Reported values represent mean ± standard deviation

	n	Soft tissue (%)	Bone (%)
Single species	9	55.88 ± 9.81	43.24 ± 9.92
Cattle	5	49.67 ± 8.20	49.62 ± 8.14
Poultry	1	59.48	39.20
Swine	3	65.01 ± 4.71	33.96 ± 7.61
Mixed species	9	66.91 ± 7.62	31.94 ± 7.68
All samples	18	61.39 ± 10.24	37.59 ± 10.38

Table 3. Proximate composition of dry, defatted MBM fractions, across all samples. Reported values represent mean ± standard deviation ($n = 18$)

	Crude protein (%) (N × 6.25)	Non-lipid organic matter (%)	Ash (%)
Soft tissue particles	81.01 ± 5.55	89.08 ± 2.32	10.92 ± 2.32
Bone particles	30.69 ± 1.15	35.78 ± 1.29	64.22 ± 1.29

The proximate compositions of the soft tissue and bone fractions are quite different from each other but are fairly consistent regardless of species (Table 3). Because the compositions of the fractions vary little, almost all of the variation in overall MBM protein and ash composition is due to the variation in proportions of the two fractions. This finding is consistent with earlier work[11] demonstrating that calcium and phosphorus content of MBM can be accurately estimated by measuring the volume of the bone fraction from a given sample.

The reported values for *crude protein* may differ significantly from the *true protein* content because the conventional nitrogen-to-protein conversion factor of 6.25 is unlikely to be accurate. This conversion factor depends on the total amino acid composition of the protein being tested; the total amino acid composition of the bone and soft tissue fractions is not known. Accurate conversion factors for beef and chicken muscle have been reported (5.72 and 5.82, respectively),[16] but it is not clear that the soft-tissue fraction of MBM has a composition similar to muscle. Comparing the amino acid composition of beef muscle to a partial amino acid profile of beef bone suggests that these two tissue types would have significantly different conversion factors. Therefore, the measure of crude protein can be used to estimate the variability in protein content between samples and to estimate the true protein content very roughly. The content of non-lipid organic matter (NLOM) reported in Table 3 may be a better measure of true protein. This measure, however, also includes small amounts of non-protein substances such as glycosaminoglycans, known to be present in bone tissue.[17] Using mean values from Tables 2 and 3, and using NLOM as the estimate of protein content, it can be shown that >80% of all MBM protein is in the soft tissue fraction.

Further investigation of MBM protein utilized only a subset of our samples that were produced from a single species: two cattle, two swine, and two chicken samples. One of the chicken samples was reported to be 99% chicken and the other was reported to be 100% poultry, species undefined; this second sample was obtained later and not included in the preceding results.

Protein type

Knowledge of the relative abundance of the different protein types that comprise MBM protein would be quite valuable in utilization research, but this cannot be determined by conventional molecular biological techniques, due to the very low solubility of MBM protein. The single most abundant protein group in MBM is very likely the collagens. The collagens comprise 25–30% of total body protein in mammals,[18] including 90–95% of all bone protein,[19–22] the majority of protein in skin, tendon and loose connective tissue, and 5% of muscle protein.[23]

The concentration of collagens in a sample can be estimated by analyzing the concentration of the non-standard amino acid 4-hydroxyproline. A large majority of hydroxyproline in higher animals is found in the collagens, although the protein elastin also contains small amounts. Hydroxyproline-to-collagen conversion factors are mentioned occasionally in the literature,[22] but an extensive literature search failed to find an explicit basis for these factors. For the present work, we used previously published amino acid analyses to determine the percentage, by mass, of 4-hydroxyproline in several types of collagen (Table 4). Rather than attempt to use different factors for the various types of MBM samples and fractions, we chose to use the median of the values reported in Table 4 (0.122) as the conversion factor for all analyses.

The amount of collagen present is reported as a percentage of the sample's NLOM. This unusual metric is used because of our low confidence in crude protein as a measure of the true protein content, as discussed above. The collagen content determined for the bone particles (Table 5) was much lower than anticipated, based on the known collagen content of raw bone tissue. Two main hypothetical phenomena may account for this discrepancy. It will be shown in the next section that a significant fraction of MBM protein exists as small, soluble peptides, presumably produced by hydrolysis of the native proteins of the raw tissue. Small fragments of collagen may diffuse during the rendering process away from their high concentration in bone particles and infiltrate soft tissue particles. It is also possible that these small peptides are lost in the sample preparation procedure used for the bone samples; the gel filtration used to separate the EDTA from the soluble bone proteins would have also separated out small peptides.

Table 4. Hydroxyproline-to-collagen conversion factors calculated using amino acid analysis of collagen from a variety of tissues

	Conversion factor	Based on data from
Cattle		
Intramuscular connective tissue	0.135	39
Tendon	0.118	40
Skin	0.114	40
Chicken		
cartilage	0.120	41
Skin	0.131	42
Bone	0.122	43
Swine		
Intramuscular connective tissue	0.126	39

Table 5. Percentage of non-lipid organic matter that is collagen. Reported values represent mean ± standard deviation

	n	Soft tissue	Bone
All species	6	16.72 ± 2.47	24.96 ± 3.88
Cattle	2	18.50 ± 1.16	28.24 ± 1.93
Poultry	2	17.08 ± 0.52	20.28 ± 1.22
Swine	2	14.59 ± 3.64	26.36 ± 1.04

Protein solubility

Previous research utilizing the same MBM samples found that in the median sample only 5.4% of MBM protein was soluble under standardized mild conditions;[1] no sample had greater than 7.2% solubility. In the present study, each particle type was extracted with either a dilute salt solution or with a solution designed to be very aggressive in solubilizing protein (Table 1). The amount and types of protein extracted with this more aggressive solution are intended to indicate the practical upper limit for non-hydrolytic functional protein extraction. The results show that the majority of MBM protein is not soluble (Table 6). The aggressive solution extracted much more protein than the mild solution, and both solutions were able to extract a greater proportion of the protein in the soft tissue than in the bone. Although there were significant differences in solubility between species, the differences were not entirely consistent. In many cases, the poultry protein was the most soluble.

Several studies[24–27] have found that the meats of various species, both raw and cooked, can be distinguished by the band patterns their extracts produce on an SDS-PAGE gel. Examination of band patterns generated by our MBM protein extracts (Fig. 1) does not reveal obvious and consistent species-to-species differences. The difference in the patterns generated by the mild and aggressive extractions suggests that the many different proteins in MBM have different solubility characteristics. It is likely that all protein types that are soluble using the mild solution are also soluble using the aggressive solution, but that some bands from the mild solution extract are not visible in the aggressive solution extract due to a technical limitation of the technique. The extract from the mild solution had to be loaded onto the gel at a greater concentration in order to produce visible bands. In the aggressive solution extract, the same proteins are probably present but not visible because they are at a low concentration.

The majority of the extracted protein does not appear within one of the few, well-defined bands on the SDS-PAGE gels; most is in the relatively uniform areas between the bands. In

Table 6. The proportion of MBM protein, estimated by NLOM, that is soluble under mild or aggressive solubilizing conditions. In all cases, two samples from each species were analyzed. Reported values represent mean ± standard deviation

	Soft tissue	Bone
Aggressive extraction		
All species	34.1 ± 5.3	13.8 ± 3.4
Cattle	27.2 ± 2.4	9.1 ± 2.2
Poultry	51.1 ± 4.3	21.6 ± 1.8
Swine	24.0 ± 1.8	10.9 ± 1.9
Mild extraction		
All species	14.4 ± 2.2	4.2 ± 1.4
Cattle	17.9 ± 1.1	2.3 ± 1.0
Poultry	14.7 ± 1.5	6.7 ± 0.7
Swine	10.7 ± 1.1	3.6 ± 0.6

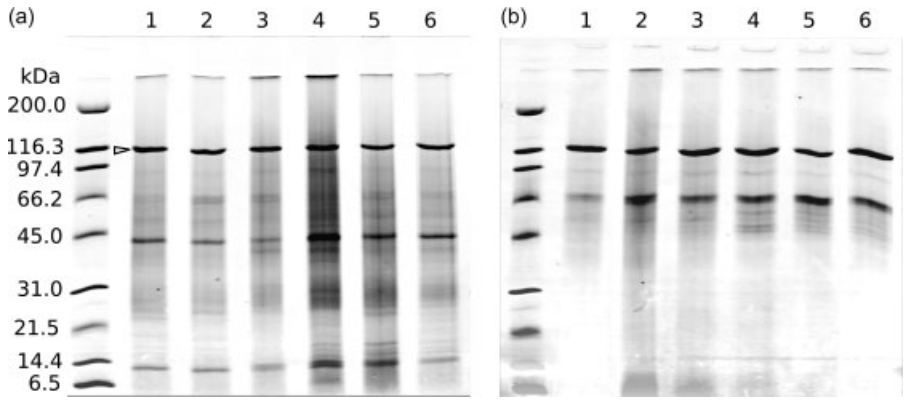

Figure 1. MBM soft tissue particles extracted with either (a) aggressive extraction solution or (b) mild extraction solution, and run on an 8–25% gradient SDS-PAGE gel. Lanes 1 and 2 represent extracts from two different MBM samples made entirely from cattle tissue. Similarly, lanes 3 and 4 and lanes 5 and 6 are from poultry and swine-derived MBM, respectively. The triangle symbol indicates the position of the internal standard.

past studies, electrophoretic patterns produced by muscle tissue extracts changed progressively depending on the intensity of the heat treatment received by the tissue; individual protein bands became weaker and background staining became more prominent. One group[28,29] observed a strong linear correlation between the heat treatment of pork muscle and decreased intensity of the actin band on an SDS-PAGE gel; the pattern for tissue heated to 133 °C (3 bar, 20 min) is a featureless streak. Extracts from MBM similarly produced nearly featureless streaks in past studies.[29,30] This simultaneous decrease in band intensity and increase in background staining is usually interpreted as the result of heat-induced, random protein hydrolysis. Past results, however, do not show an obvious shift in the staining pattern towards the lower molecular mass (M_r) region of the gel, as would be expected based on this interpretation. Other groups' results[31,32] suggest that heating results in a progressive random aggregation of relatively high M_r proteins in cattle and horse muscle, rather than protein hydrolysis. This interpretation is consistent with phenomena we observed. In early experiments (not shown) there were large amounts of proteinaceous material of very high apparent M_r; darkly stained areas occurred where the extract was loaded onto the gel as well as at the boundary between the stacking gel and the main gel. Ultrasonic treatment of the extracts prior to SDS-PAGE simultaneously minimized these features and increased the intensity of the distinct bands. We hypothesize that the continuous M_r distribution of MBM protein results from a combination of this heat-induced aggregation and *enzyme-catalyzed* hydrolysis, rather than heat-induced hydrolysis. Peptides resulting from enzymatic hydrolysis are known to increase as raw meat is stored.[33,34] It is likely that similar hydrolysis occurs in raw tissue bound for rendering which is stored for a much shorter time (<1 day), but at much warmer temperatures.

Molecular mass distribution of soluble protein

The overall molecular mass distribution (MMD) of the extracted protein is a key factor in the functional utilization of this protein; SDS-PAGE gives some indication of this distribution. The relative distance from the origin traveled (R_f) by an analyte molecule is related to M_r (Fig. 2(a)). The fluorescent intensity (I) at a section on the gel is directly proportional to the mass of protein in that section of the gel; R_f versus I can be plotted in an electropherogram such as Fig. 2(b), so that the area under a section of the trace has direct proportionality to protein mass. This figure, however, gives a distorted impression of the extract's MMD for a number of reasons related to the non-linear relationship between R_f and M_r. In addition to the non-linearity of the scale, and the counterintuitive placement of low M_r at the right-hand side of the scale, this figure gives a *very* misleading impression of the amount of protein in any particular size range. Since any particular gel can only resolve a limited range of M_r, multiple gels are required to obtain a complete picture of the extract's MMD; in the present study, each extract was run on two types of gels optimized for different M_r ranges (compare Fig. 2(a) and (c)). The data from two different gel types (Fig. 2(b) and (d)), however, cannot be combined by simply placing the two sets side by side.

To address these issues and present our MMD data in an undistorted, intuitive format, we developed a method for transforming the data. The x-axis is made more intelligible by replacing R_f values with the corresponding M_r values according to the standard curve

$$\log_{10} M_r = Q \cdot R_f + P \quad (1)$$

where P and Q are determined by fitting the curve to data from a range of M_r standards run on the same gel. This replacement of R_f with M_r, however, destroys the proportionality between area under the trace and mass of protein. It can be shown that this proportionality is restored by replacing I with I' according to

$$I' = -I\left[\frac{0.434}{Q \cdot M_r}\right] \quad (2)$$

The transformed and combined data (Fig. 2(e)), while presenting equivalent data, give a different impression. The black bars below the x-axis in Fig. 2(a) and (e) encompass the same data points and highlight the distorted presentation of the data in its original form. Only in Fig. 2(e) does it become apparent that the majority of the mass of protein exists as proteins and peptides of less than 65 kDa. Figure 3 presents data transformed in this manner from four different extracts of the same MBM sample. Comparison of Fig. 3(a) and (b) demonstrates an additional benefit of this data presentation format; the difference in overall amount of protein extracted by the mild and aggressive solutions is much more apparent. On the gel, the concentration of the mild and aggressive extracts had to be adjusted differently in order to

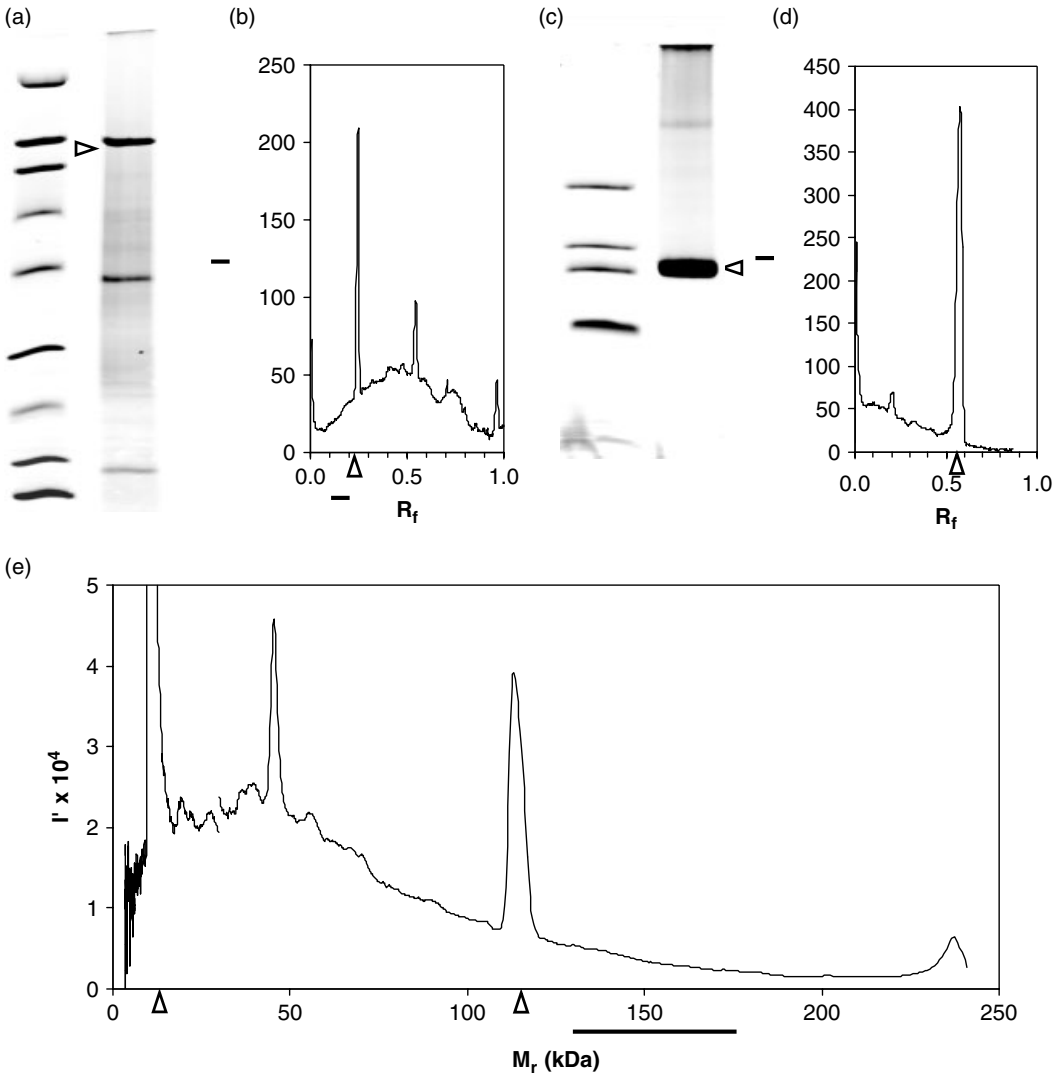

Figure 2. Molecular mass distribution of protein extracted from the soft tissue fraction of bovine MBM using the aggressive extraction solution. The extract was analyzed by SDS-PAGE on (a) 8–25% polyacrylamide and (c) high-density gels. Fluorescence intensity *versus* relative migration distance data from these gels is plotted in b and d, respectively. Data from both gels, transformed as described in the text, are replotted (e) in terms of molecular mass. In each instance, a triangle symbol indicates the position of an internal standard.

put each in the range useable by the gel technique. The plots in Fig. 3 reflect the original concentrations of the extracts.

The overall MMD plot for the same type of extract from different samples (e.g., bone fraction from two different samples extracted with the mild solution) was qualitatively similar. The differences in MMD can be examined more quantitatively using metrics common to the field of polymer science. Mass-average molecular mass ($\overline{M}_w$) and number-average molecular mass ($\overline{M}_n$) are measures of central tendency, which together determine the polydispersity (PD), a measure of the dispersion of the molecular masses.[35,36] These values from the MBM protein extracts (Table 7) reveal that soluble MBM protein is highly polydisperse, a potential difficulty for functional utilization. The results presented here do not include peptides smaller than about 2 kDa that may have been present, due to the limitations of the techniques employed. Presence of such small peptides could account for our measurement of less protein on the gels than would be expected from our measurements of total protein concentration in the extracts. Size exclusion chromatography or multi-angle light-scattering analysis might serve better than SDS-PAGE in producing a complete characterization of an extract's MMD.

In conclusion, MBM protein is a challenging substance to utilize functionally; it is heterogeneous at macro- and microscopic scales, poorly soluble, and interspersed with non-protein substances. An important step on the path towards new commercial utilization will be the processing of MBM into a form that is more uniform, soluble, and enriched in protein. Our finding that the soft tissue fraction contains more than 80% of MBM's protein and that this protein is more soluble than the protein of bone fraction suggests that MBM should be fractionated[37] before it is treated to extract protein; the low-protein bone fraction could be utilized for other applications such as phosphorus fertilizer. Our finding of the low solubility of MBM protein, even under the most aggressive non-hydrolytic conditions, suggests that limited hydrolysis,[38] to increase solubility while retaining some functionality, will be required. Our finding that a large portion of MBM protein is collagen suggests that an extract of MBM protein will have some of the functional properties of gelatin.

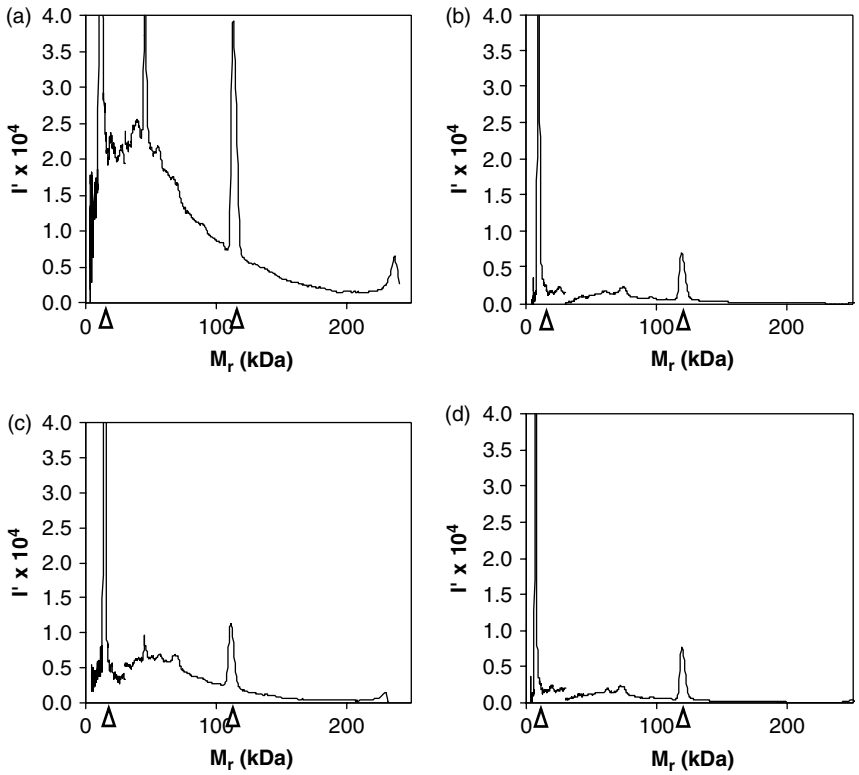

Figure 3. Molecular mass distribution of proteins and peptides extracted from bovine MBM. The soft-tissue fraction of the MBM was extracted with either (a) aggressive or (b) mild extraction solution. Similarly, the bone fraction was extracted with either (c) aggressive or (d) mild extraction solution. The break in each trace at 30 kDa is a result of combining the data from two different gel types. In each instance, the triangle symbol indicates the position of an internal standard.

Table 7. The molecular mass distribution of MBM extracts in terms of mass-average molecular mass ($\overline{M}_w$), number-average molecular mass ($\overline{M}_n$), and polydispersity (PD). Underlying data were corrected to eliminate influence of the internal standards on these values. In all cases, two samples from each species were analyzed. Reported values represent mean ± standard deviation

	Aggressive extraction			Mild extraction		
	$\overline{M}_w$ (kDa)	$\overline{M}_n$ (kDa)	PD	$\overline{M}_w$ (kDa)	$\overline{M}_n$ (kDa)	PD
Soft tissue						
All species	71.1 ± 4.6	32.4 ± 6.4	2.24 ± 0.32	71.2 ± 18.1	39.4 ± 17.5	2.07 ± 0.76
Cattle	70.7 ± 0.7	32.8 ± 0.1	2.16 ± 0.03	69.2 ± 0.7	42.5 ± 6.4	1.65 ± 0.27
Poultry	76.1 ± 2.9	38.8 ± 5.6	1.97 ± 0.21	87 ± 3.0	56.1 ± 1.7	1.55 ± 0.01
Swine	66.4 ± 2.4	25.6 ± 1.2	2.60 ± 0.21	57.4 ± 27.2	19.6 ± 11.3	3.02 ± 0.35
Bone						
All species	74.0 ± 11.3	29.6 ± 10.3	2.69 ± 0.73	86.7 ± 5.4	59.8 ± 11.3	1.49 ± 0.26
Cattle	82.8 ± 8.4	39.3 ± 5.7	2.11 ± 0.09	80.5 ± 5.2	54.8 ± 0.9	1.47 ± 0.07
Poultry	74.0 ± 8.3	29.7 ± 7.0	2.53 ± 0.32	89.7 ± 0.9	50.8 ± 5.5	1.78 ± 0.17
Swine	65.1 ± 13.7	20.0 ± 8.8	3.44 ± 0.83	90.1 ± 0.7	73.9 ± 0.8	1.22 ± <0.01

ACKNOWLEDGEMENTS

Bill Damert, Kelly Grube, Robert DiCiccio, Brian Coll, Laurie Fortis, Michael Tunick and Chad Mazenko provided assistance that was valuable to the completion of this research.

REFERENCES

1 Garcia RA, Rosentrater KA and Flores RA, Characteristics of North American meat and bone meal relevant to the development of non-feed applications. *Appl Eng Agric* **22**:729–736 (2006).
2 Garcia RA, Onwulata CI and Ashby RD, Water-plasticization of extruded material made from meat and bone meal and sodium caseinate. *J Agric Food Chem* **52**:3776–3779 (2004).
3 McDonnell K, Desmond J, Leahy JJ, Howard-Hildige R and Ward S, Behaviour of meat and bonemeal/peat pellets in a bench scale fluidised bed combustor. *Energy* **26**:81–90 (2001).
4 Lazarovits G, Conn KL and Potter J, Reduction of potato scab, verticillium wilt, and nematodes by soymeal and meat and bone meal in two Ontario potato fields. *Can J Plant Pathol* **21**:345–353 (1999).
5 Wescott JM, Frihart CR and Traska AE, High-soy-containing water-durable adhesives. *J Adhes Sci Technol* **20**:859–873 (2006).

6 Hojilla-Evangelista MP, Adhesive qualities of soybean protein-based foamed plywood glues. *J Am Oil Chem Soc* **79**:1145–1149 (2002).
7 Kolomaznik K, Mladek M, Langmaier F, Janacova D and Taylor MM, Experience in industrial practice of enzymatic dechromination of chrome shavings. *J Am Leather Chem Assoc* **94**:55–63 (1999).
8 Taylor MM, Cabeza LF, DiMaio GL, Brown EM, Marmer WN, Carrió R, *et al*, Processing of leather waste: pilot scale studies on chrome shavings. Part I. Isolation and characterization of protein products and separation of chrome cake. *J Am Leather Chem Assoc* **93**:61–82 (1998).
9 Tomasula PM, Parris N, Yee W and Coffin D, Properties of films made from CO_2-precipitated casein. *J Agric Food Chem* **46**:4470–4474 (1998).
10 Dangaran KL, Cooke P and Tomasula PM, The effect of protein particle size reduction on the physical properties of CO_2-precipitated casein films. *J Food Sci* **71**:E196–E201 (2006).
11 Mendez A and Dale N, Rapid assay to estimate calcium and phosphorus in meat and bone meal. *J Appl Poult Res* **7**:309–312 (1998).
12 Van Wandelen C and Cohen SA, Using quaternary high-performance liquid chromatography eluent systems for separating 6-aminoquinolyl-*n*-hydroxysuccinimidyl carbamate-derivatized amino acid mixtures. *J Chromatogr A* **763**:11–22 (1997).
13 Nishihara JC and Champion KM, Quantitative evaluation of proteins in one- and two-dimensional polyacrylamide gels using a fluorescent stain. *Electrophoresis* **23**:2203–2215 (2002).
14 Ahnert N, Patton WF and Schulenberg B, Optimized conditions for diluting and reusing a fluorescent protein gel stain. *Electrophoresis* **25**:2506–2510 (2004).
15 Christiansen P, Mass allometry of the appendicular skeleton in terrestrial mammals. *J Morphol* **251**:195–209 (2002).
16 Sosulski FW and Imafidon GI, Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods. *J Agric Food Chem* **38**:1351–1356 (1990).
17 Pugliarello MC, Vittur F, de Bernard B, Bonucci E and Ascenzi A, Chemical modifications in osteones during calcification. *Calcif Tissue Int* **5**:108–114 (1970).
18 Junquiera LC and Carneiro C, *Basic Histology* (10th edn). Lange, New York (2003).
19 Zarkadas CG, Yu Z, Zarkadas GC and Minero-Amador A, Assessment of the protein quality of beefstock bone isolates for use as an ingredient in meat and poultry products. *J Agric Food Chem* **43**:77–83 (1995).
20 Franzen A and Heinegard D, Extraction and purification of proteoglycans from mature bovine bone. *Biochem J* **224**:47–58 (1984).
21 Eastoe JE and Eastoe B, The organic constituents of mammalian compact bone. *Biochem J* **57**:453–459 (1954).
22 Dennison KJ, Amino acids in archaeological bone. *J Archaeol Sci* **7**:81–86 (1980).
23 Eastoe JE, The amino acid composition of mammalian collagen and gelatin. *Biochem J* **61**:589–600 (1955).
24 Pineiro C, Barros-Velazquez J, Perez-Martin RI, Martinez I, Jacobsen T, Rehbein H, *et al*, Development of a sodium dodecyl sulfate–polyacrylamide gel electrophoresis reference method for the analysis and identification of fish species in raw and heat-processed samples: a collaborative study. *Electrophoresis* **20**:1425–1432 (1999).
25 Ozgen Arun O and Ugur M, Animal species determination in sausages using an SDS-PAGE technique. *Arch Lebensmittelhyg* **51**:49–53 (2000).
26 Bauer F and Hofmann K, Electrophoretic identification of animal species in heat treated meat and meat products. *Fleischwirtschaft* **69**:419–422 (1989).
27 Hofmann K, Fundamental problems in identifying the animal species of muscle meat using electrophoretic methods. *Fleischwirtschaft* **67**:820–826 (1987).
28 Hofmann K, Fischer K, Muller E and Kemper V, Experiments to demonstrate the effectiveness of heat treatments applied to canned meats and meat-and-bone meals. *Fleischwirtschaft* **76**:920–923 (1996).
29 Hofmann K, Proof of proper heating at meat-and-bone meals. *Fleischwirtschaft* **76**:1037–1039 (1996).
30 Kim S-H, Huang T-S, Seymour TA, Wei C-I, Kempf SC, Bridgman CR, *et al*, Identification of a biomarker for the detection of prohibited meat and bone meal residues in animal feed. *J Food Sci* **69**:C739–C745 (2004).
31 Lee YB, Rickansrud DA, Hagberg EC and Eriskey EJ, Application of SDS-acrylamide gel electrophoresis for determination of the maximum temperature to which bovine muscles have been cooked. *J Food Sci* **39**:428–429 (1974).
32 Babiker SA, Glover PA and Lawrie RA, Improved methodology for the electrophoretic determination of horse meat in heated foodstuffs. *Meat Sci* **5**:473–477 (1981).
33 Sylvestre MN, Feidt C and Brun-Bellut J, Post-mortem evolution of non-protein nitrogen and its peptide composition in growing lamb muscles. *Meat Sci* **58**:363–369 (2001).
34 Bauchart C, Remond D, Chambon C, Patureau Mirand P, Savary-Auzeloux I, Reynes C, *et al*, Small peptides (<5 kDa) found in ready-to-eat beef meat. *Meat Sci* **74**:658–666 (2006).
35 Cowie JMG, *Polymers: Chemistry and Physics of Modern Materials* (2nd edn). CRC Press, Boca Raton, FL (1991).
36 Bootz A, Russ T, Gores F, Karas M and Kreuter J, Molecular weights of poly(butyl cyanoacrylate) nanoparticles determined by mass spectrometry and size exclusion chromatography. *Eur J Pharm Biopharm* **60**:391–399 (2005).
37 Garcia RA, Flores RA and Phillips JG, Use of an aspirator to separate meat and bone meal into high ash and high protein streams. *Trans ASAE* **48**:703–708 (2005).
38 Coll BA, Garcia RA and Marmer WN, Diffusion of protease into meat and bone meal for solubility improvement and potential inactivation of the BSE prion. *PLoS One* **2**:e245 (2007).
39 McClain PE, Wiley ER and McCague KE, Species variation in the cross-linking characteristics of collagen from the intramuscular connective tissues of striated muscles (*Bos taurus, Ovis aries, Sus scrofa*). *Int J Biochem* **2**:167–172 (1971).
40 Piez KA and Linkins RC, The nature of collagen. II. Vertebrate collagen, in *Calcification in Biological Systems*, ed. by Sognnaes RF. American Association for the Advancement of Science, Washington, DC, pp. 411–420 (1960).
41 Trelstad RL, Kang AH, Igarashi S and Gross J, Isolation of two distinct collagens from chick cartilage. *Biochemistry* **9**:4993–4998 (1970).
42 Kang AH, Piez KA and Gross J, Characterization of the alpha chains of chick skin collagen and the nature of the NH_2-terminal cross-link region. *Biochemistry* **8**:3648–3655 (1969).
43 Francois CJ and Glimcher MJ, The isolation and amino acid composition of the [alpha] chains of chicken-bone collagen. *Biochim Biophys Acta, Protein Structure* **133**:91–96 (1967).